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NaCl Toxicity Testing in Rice Cultivars at the Seedling Stage: Evaluating the Applicability of IC_{50} as a Method using Hydroponic Medium

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Abstract: **Salt tolerant cultivars of rice are continuously being developed for suitability of their cultivation in salt affected lands that cause significant loss in the crop yield. Testing of salt tolerance of a large number of breeding lines generated after crossing and selfing is a major constrain in the development of salt tolerant cultivars. The present study describes a laboratory based method of testing salt tolerance of the breeding lines at the seedling stage. Shoot growth inhibition data were generated for five rice cultivars after hydroponic exposure of the 6 days old seedlings to 0.5%, 1.0%, 1.5 %, and 2.0% for 24, 48, and 72 h. Tukey-Kramer's test revealed a significant difference in shoot growth inhibition between treatment concentrations for each of the cultivars. The IC_{50} values for NaCl varied from 153 mM (0.5%) for the salt-sensitive Naveen to 284 mM (1.5%) for the salt-tolerant Pokkali. IC_{50} values demonstrated a difference in salt-tolerance/sensitivity of the rice cultivars, and hence the method can be used for testing salt tolerance of the breeding lines in the shortest possible time. Moreover, the method provides a quantitative value, rather than mere phenotypic observations being followed in the prevailing method of screening of salt tolerance of breeding lines.**

Index Terms: **IC_{50}, NaCl toxicity, Oryza sativa, Rice seedling, Salt tolerance.**

I. INTRODUCTION

Rice belongs to the family Gramineae or Poaceae, and is widely accepted as a staple food crop for more than 3 billion people worldwide, and accounts for 50-80% of total caloric energy intake by individuals (Khush, 2005). It contributes to 1/3rd of total carbohydrate source and serves as a nutrient supplement for Zinc and Niacin (Vitamin B3) (Gopalan et al., 2007). It is expected that the population of the world is to cross 9 billion by the year 2050 (Cominelli et al., 2013), and we need to produce 70% more food to sustain the increased population (FAO, 2009). Accordingly, rice production has to be increased to at least 800 million tons from the current production of approximately 500 million tons (Virk et al., 2004). The challenge is despite the fact that rice is second to wheat in areas over which these crops are cultivated; 214 million ha/year for wheat compared to 140 million ha/year for rice (Macovei et al., 2012). Furthermore, the increase in the food supply, including that of rice, has to come only from the current arable lands, as there is little potential to bring more lands into cultivation (WWD, 2012). With regard to rice, improvements in its yield through nutrients management appears to be saturated, and a further increase in its yield is envisioned through a breeding program directed towards the reduction in the loss inflicted upon by abiotic stresses (Cominelli et al., 2013; Wu, 2018; Arzani, 2008; Evangelista et al., 2013).

Environmental factors affect crop yield greatly. According to one estimate, abiotic stresses are responsible for more than 60% yield loss of major crops worldwide (Bray & Bailey-Serres, 2000). Salinity is second only to drought among the environmental stresses that cause significant loss in agricultural production (Khavari-Nejad et al., 2008; Lutts et al., 1995). Approximately 955 million hectares of agricultural land worldwide is salt affected (Subbarao et al., 1990), which is more than 6% of the global land (Rekha et al., 2018), including 45 million hectares of irrigated lands the world over (Boyer, 1982; Rengasamy, 2010). According to UNEP (United Nations Environment Program), 50% of cropland and 20% of agricultural land are salt affected (Li, 2008). So far as rice is concerned, millions of hectares of land in the humid regions of South and

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Southeast Asia suitable for cultivation of the crop are cultivated with very low yield because of salinity problem, including 12 million ha of land area in Asia alone (Kumar et al., 2015). Besides, more and more agricultural lands are getting increasingly saline because of the use of fertilizers and poor quality of water for irrigation (Szabolcs, 1977; Nelson et al., 1998). It is expected that at the current rate of secondary salinization the loss of agricultural land may be up to 50% by the middle of the 21st century (Wang et al., 2003). Salinity is a major bottleneck in the attempt of the farmers and the scientific community to increase rice production, and increasing salinization of the agricultural land is a further threat to it. Therefore, a breeding program oriented towards the development of salt tolerant lines of rice is being considered as an important strategy to increase rice production in the salt affected land, particularly in the saline prone coastal areas.

The toxic effect of salinity on rice is dependent on the growth stage at which it is exposed to stress. Several workers have reported that rice is most sensitive to salinity at the seedling stage compared to the germination, vegetative and reproductive stages (Rad et al., 2012; Lutts et al., 1996; IRRI, 1967). Hence, it is essential that screening of the genotypes being developed by the breeders for salt tolerance is done at the seedling stage, as the genotype tolerant at the seedling stage would also be tolerant to salinity at the later phase of the life cycle. Screening of germplasm for salt tolerance at the seedling stage is in practice. The criteria used for screening are visual salt-induced effects like growth (height), leaf injury, and seedling survival over two weeks (Gregorio et al., 1997; Islam and Karim, 2010; Mondal and Borromeo, 2016). However, the screening tests do not provide any quantifiable unit of salt toxicity or tolerance. The present paper explores if the concentration of NaCl that inhibits 50% shoot growth, i.e. inhibitory concentration fifty (IC_{50}), could be used in testing salt tolerance in rice genotypes as a rapid testing technique in less possible time than being followed, and without disturbing the standard growth condition set by IRRI (Gregorio et al., 1997).

II. MATERIALS AND METHODS

A. Rice seedlings growth conditions

Seeds of the rice (*Oryza sativa* L. spp. Indica) cultivars, including Pokkali, Lunishree, Luna Shankhi, Swarna, and Naveen, were collected from the germplasm center of the National Rice Research Institute, Cuttack, Odisha. The seeds were washed properly in MilliQ water 4-5 times and soaked in the same water for 24 hrs. The soaked seeds of the individual cultivars were transferred separately on a thin cotton bed moistened with Milli-Q water and housed in petriplates. The petriplates containing the seeds were covered and incubated at 30°C for 48 h hour in dark in an incubator to allow them to germinate.

The germinated seeds were grown in hydroponic system. The seeds of the individual cultivars were placed separately on nylon net wrapped around glass bangles, each of which was placed individually on top of the plastic glass of ~200 mL volume. The individual glasses were filled with Hoagland's solution, which just touched the net. The concentration and composition of nutrients in the Hoagland's media were 0.81 mM $MgSO_4.7H_2O$, 1.4 mM KNO_3, 0.499 mM Ca $(NO_3)_2.4H_2O$, 0.108 mM KH_2PO_4, 20 μM $FeCl_3$.EDTA, 9.04 μM H_3BO_3, 1.82 μM $MnCl_2.4H_2O$, 0.1391 nM $ZnSO_4.7H_2O$, 35.2 nM Na_2MoO_4 and 80 nM $CuSO_4.5H_2O$. The glasses were placed inside a plant growth chamber and maintained at 25 ± 2 °C and relative humidity of 75%. The seedlings were illuminated (16/8 h light/dark cycle) with white fluorescent light (200 μmol m-2 s-1). Fresh Hoagland's solution was added every day in the morning to make-up the loss due to evaporation and transpiration.

B. Salinity treatment to the rice seedlings

Salinity treatment was applied in the form of NaCl through roots after 5 days of germination, i.e. on the 6th day in the morning. The NaCl treatment concentrations were 0.5% (85 mM), 1.0% (170 mM), 1.5% (256 mM) and 2.0% (342 mM). The seedling not receiving NaCl treatment served as control. NaCl was added to the individual beakers as a stock solution. Initially, NaCl was added to each beaker (other than those served as a control) in the morning in a minute amount to raise the NaCl level approximately to 0.25% (42.5 mM), and the seedlings were kept in dark for half an hour to prevent the seedlings from an initial shock. More NaCl stock solution was added to the individual beakers to raise the concentration to the desired level. The light was turned on after half an hour of the final treatment to prevent the seedlings from oxidative stress. The exposure continued for the next 72 h at the stated light and dark cycle.

C. Scatter plot graph for IC_{50} dose estimation

Shoot length of the individual seedlings in each glass, including control, 0.5 % NaCl, 1.0% NaCl, 1.5% NaCl, 2.0% NaCl and 2.5% NaCl, was measured using a ruler at 0, 24, 48 and 72 h of exposure period. For the IC_{50} estimation, shoot length of a total of 24 randomly sampled seedlings from each glass was measured for the control and each treatment after 72 h of salt application. A scattered plot was drawn between the inhibition in shoot growth and the NaCl treatment doses. The concentration of NaCl inhibiting 50% of shoot growth, IC_{50}, was calculated using the algebraic equation of $y = mx + C$, where y represents the 50% average length of the control shoot, 'm' is the slope value, 'c' is the constant, and 'x' is the estimated IC_{50} value. One-Way ANOVA test was applied on the 72 h shoot length data of the randomly selected 24 seedlings of each cultivar to know if the NaCl treatment had a statistically significant effect on the shoot length of seedling of the individual rice cultivars. Tukey-Kramer's test was applied to know if the difference in inhibition of shoot growth of the seedlings of the individual cultivars was statistically significant between the two treatment concentrations. IC_{50} values of NaCl calculated were validated for their effectiveness using the fresh batch of seedlings. Five days old seedlings of each cultivar grown on the hydroponic medium stated above were treated with the NaCl concentration equivalent to the respective IC_{50} value. Shoot length of the seedlings was measured at 0 h and after 72 h of exposure to NaCl for each cultivar, as described above, and percent inhibition in the growth was calculated.

III. RESULTS AND DISCUSSION

A. Shoot growth observation for IC_{50} estimation

Hydroponic treatment of the 5 days old seedlings with 0, 85 mM (0.5%), 170 mM (1.0%), 256 mM (1.5%) mM and 342 mM (2.0%) NaCl showed varied response in different cultivars used in the study (Fig. 1). However, the inhibition in the seedling growth, measured in terms of the height of the seedlings, was most significant between all the treatment concentrations in all the cultivars, as reflected from the Tukey's-Kramers's test (Table 1). The difference in the growth inhibition was not significant between 0 to 0.5% NaCl in the salt-tolerant Pokkali, 0.5 % to 1.0 % NaCl in the salt-tolerant Lunishree and Luna Shankhi, and between 1.0 to 1.5 % NaCl in the salt-sensitive Naveen.

The response of the salt-tolerant Pokkali, Lunishree, and Luna Shankhi to the increasing NaCl treatment concentrations was more or less similar. All of them showed inhibition in shoot growth with increase in the NaCl treatment concentrations (Fig. 1). The maximum inhibition was observed at 342 mM NaCl concentration where the increase in the shoot length on different days of treatments was almost equal, showing no significant difference, except in Pokkali, which showed a significant increase in shoot length on 72 h treatment compared with that on other treatment days. The increase in shoot length in Lunishree was significantly less at 342 mM NaCl on all the treatment days compared to that in 0 mM, 24 h NaCl, which was not seen in Luna Shankhi, except on 72 h 342 mM NaCl treatment. In all the cultivars the increase in shoot length was inhibited greatly from 256 mM treatment concentration onward, but the inhibition at 256 and 342 mM concentrations was less in the salt-tolerant Pokkali compared with that in salt-tolerant Luna Shankhi and Lunishree. Despite significant inhibition in the shoot length at 256 mM 24 h treatment compared to 0 mM 24 h control in all the cultivars, the increase in the length of the shoot was significant at least on 72 h exposure. The maximum increase in the shoot length at 256 mM NaCl treatment compared to control was noticed in Pokkali, followed by in Lunishree and Luna Shankhi. Growth arrest was noticed at 256 mM NaCl treatment after 24 h treatment in Lunishree and after 24 and 48 h treatment in Luna Shankhi, which

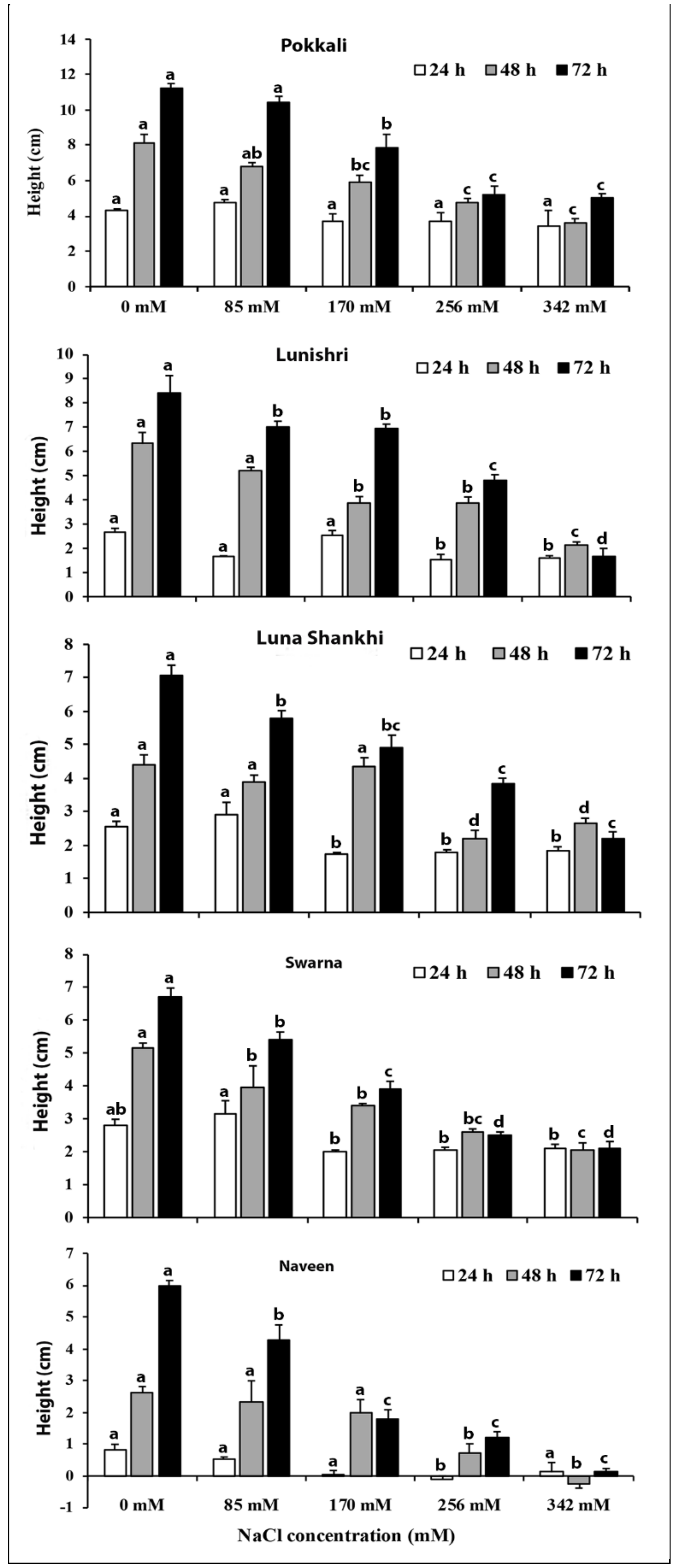

Fig. 1. Effect of different NaCl treatment concentrations (0, 85 mM or 0.5%, 170 mM or 1.0 %, 256 mM or 1.5% and 342 mM or 2.0 %) on shoot growth after 24, 48 and 72 h of hydroponic exposure. The bars representing shoot growth of the same exposure day marked with the same alphabet do not differ from each other.

was not observed in Pokkali. In general, a gradual decrease in shoot length was observed in the salt-tolerant Pokkali, Luna Shankhi, and Lunishree with increase in NaCl concentration from 0 mM to 170 mM, but in Pokkali and Luna Shankhi, the decrease on 48 h exposure was insignificant compared to the decrease that was observed in Lunishree. In contrast to the salt-tolerant cultivars, the salt-sensitive Swarna showed the arrest of growth at 256 mM and higher NaCl treatment concentrations, as there occurred no significant increase in the shoot length after 48 and 72 h exposure compared with that observed after 24 h at 256 and 342 mM NaCl concentrations. Compared with Swarna, the toxic response to NaCl was even greater in the salt-sensitive Naveen. The toxic response by the seedlings to NaCl was observed upon 24 h exposure even at 85 and 170 mM concentrations. After 24 h, however, the seedlings somewhat recovered from the treatment shock and showed a little growth up to 256 mM NaCl exposure. Nevertheless, the 48 h exposure was sufficient to inhibit the growth to the maximum at 256 mM NaCl treatment. At 342 mM NaCl, the growth of the seedlings was completely arrested, even after 48 and 72 h exposures.

The seedlings of all the cultivars showed a linear decrease in shoot length with increase in NaCl treatment concentrations measured after 72 h of exposure with a high degree of co-efficient of correlation (Fig. 2). It was possible to figure out the IC_{50} value for the inhibition of shoot length from the graph between the inhibition of shoot length and the treatment concentrations for each rice cultivars. However, for more accurate IC_{50} values of NaCl for the individual cultivars, an algebraic approach was followed. Pokkali was found to be the most NaCl tolerant cultivar among the five, as the algebraic IC_{50} value for shoot length for this cultivar was the highest, i.e., 284 mM. Luna Shankhi was found to be the 2nd most NaCl tolerant with the algebraic IC_{50} value for shoot length for this cultivar being 261 mM. The cultivar Lunishree was next to Luna Shankhi in NaCl tolerance with the algebraic IC_{50} value of 254 mM. The tolerance of Swarna to NaCl was also found to be very high with an algebraic IC_{50} value of 225 mM. The cultivar Naveen was found to be the most sensitive to NaCl, and in fact very sensitive to NaCl with algebraic IC_{50} to 153 mM.

Treatment of the individual cultivars with the respective IC_{50} NaCl concentrations revealed that shoot growth in these cultivars was in fact inhibited by at least 50 % (Fig. 3), validating the algebraically derived IC_{50} NaCl concentrations for each of the rice cultivars considered in the study. The inhibition in the shoot growth obtained at the IC_{50} NaCl concentration was a little higher than 50 % for Pokkali, Swarna, and Luna Shankhi (Fig. 3).

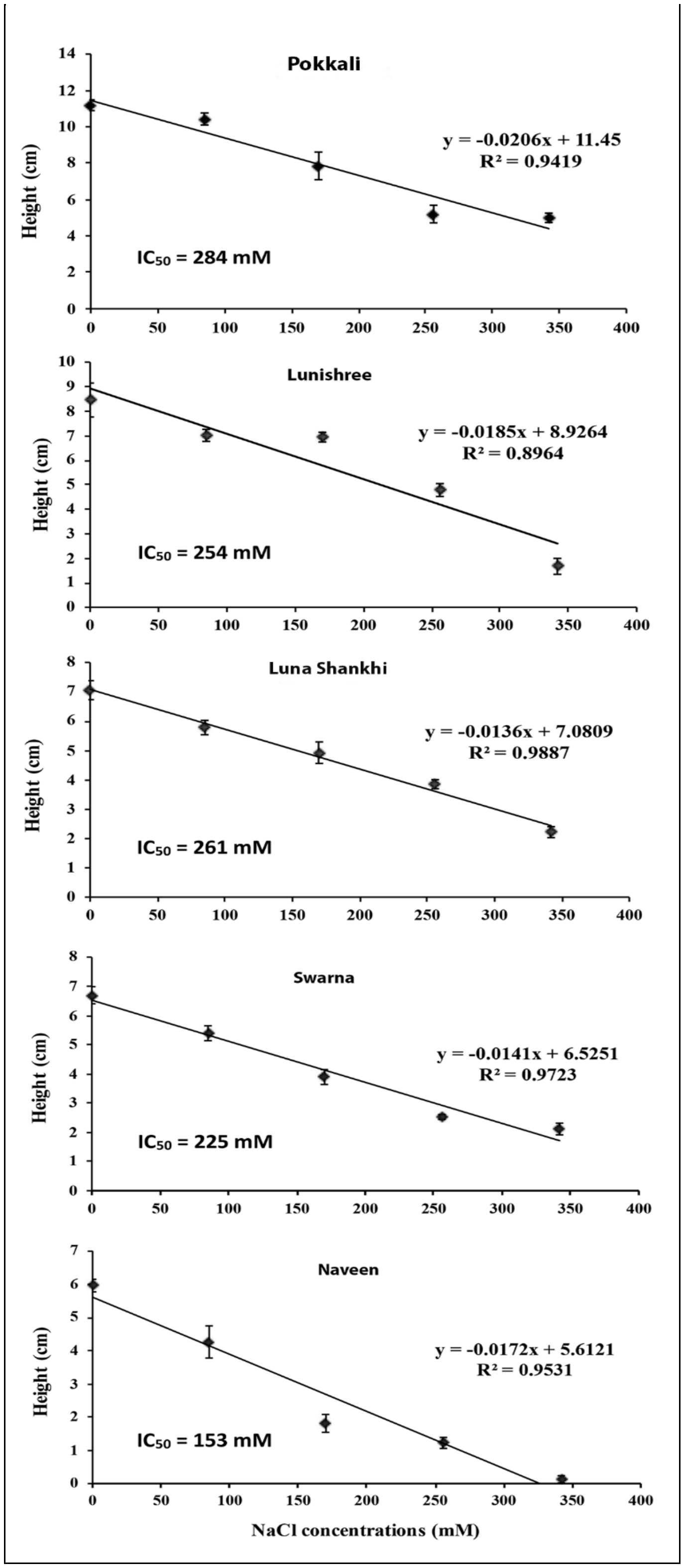

Fig. 2. IC_{50} (50% inhibitory concentration) determination for shoot length inhibition by correlation analysis between shoot growth and NaCl treatment concentrations (0, 85 mM or 0.5%, 170 mM or 1.0 %, 256 mM or 1.5% and 342 mM or 2.0 %). Effect on shoot growth after 72 h exposure was considered the shoot growth inhibition study.

B. Comparative analysis of IC_{50} for seedling shoot growth inhibition among the rice varities

Reclamation of salt affected land for agriculture purposes is not cost effective (Subasinghe et al., 2007). Hence, the effort is always on to produce salt-tolerant genotypes by breeding programs for their cultivation in salt affected land (Rekha et al., 2018). However, the challenge of such a breeding program lies in the early and easy screening of the salt-tolerant genotypes, which is cost effective and at the same time scientifically recognizable. An important parameter that is considered in screening is germination under saline conditions (Islam and Karim, 2010; Subasinghe et al., 2007). However, in the present method of investigation, the germination is not required to be done under saline conditions, which saves the experimental burden.

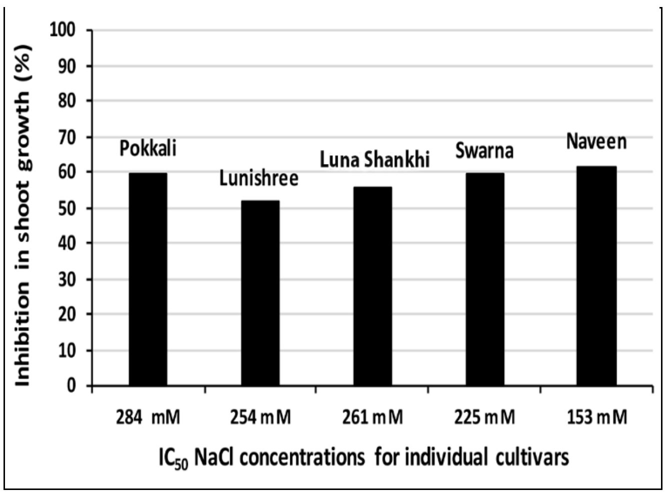

Fig. 3. Validation of the 72 h IC50 values obtained for various rice cultivars. Shoot growth inhibition of the rice cultivars at their respective algebraically derived IC50 values was checked for the purpose.

It is now well established that the seedling stage of development is the most sensitive stage to the toxic effect of salinity in rice (Rekha et al., 2018; Lutts et al., 1996; Vispo et al., 2015). Hence, phenotyping for salt tolerance of a breeding line/cultivar at the seedling stage is likely to reflect their genotype in a true sense in terms of their salt tolerance feature. The phenotyping of the seedlings of the five rice cultivars clearly distinguishes their salt tolerance feature with Pokkali being the most tolerant followed by Luna Shankhi, Lunishree, Swarna, and Naveen (Fig. 1). The shoot growth inhibition study for 72 h not only demonstrated the high salt tolerance capability of Pokkali but could also differentiated the salt tolerant capability of even two nearly similar salt-tolerant cultivars, Lunishree and Luna Shankhi. The same is also reflected from the calculation of the 72 h IC_{50} for shoot inhibition; the IC_{50} for shoot inhibition for Lunishree was 254 mM NaCl (19.05 dS/m), while that for Luna Shankhi was 261 mM (19.58 dS/m) (Fig. 2). However, the arrest in growth in both Lunishree and Luna Shankhi from 342 mM treatment concentration onward (Fig. 1) indicated that the IC_{50} value may decrease if a longer duration treatment is considered for its calculation. In contrast, the arrest of growth at 342 mM NaCl was not complete in Pokkali, indicating that the IC_{50} of Pokkali may be higher if a longer duration exposure is considered for the species. As a standard practice, for the screening of salt tolerance a treatment duration of 12 to 14 days is considered for the visual observation of injuries to the seedlings and their survival (Bado et al., 2016; Mohammadi et al., 2014; Chunthaburee et al., 2016). The visual injury symptom is given a rank score of 1 to 9, with score 1 indicating the seedlings showing normal growth and score 9 indicating the seedlings either dead or dying (Islam and Karim, 2010; Bado et al., 2016). The importance of considering a longer duration exposure for judging salt tolerance of a rice variety/cultivar is also reflected from the observation of a high 72 h IC_{50} of the salt-sensitive cultivar Swarna, 225 mM (16.85 dS/m), that is much more than 10 dS/m electrical conductivity (EC) of soil beyond which the rice yield is drastically reduced (Akbar and Senadhira, 1985). The arrest in shoot growth even at 256 mM NaCl also supports consideration of a long period of exposure to salinity for visual observation. With 72 h IC_{50} of 153 mM or 11.38 dS/m (Fig. 2) and the start of shoot growth arrest at 170 mM NaCl, the cultivar Naveen can certainly be considered as the most salt-sensitive among the cultivars used for the study. However, the IC_{50} EC is still higher than that at which the yield is drastically reduced (Akbar and Senadhira, 1985).

Table 1- Tukeys-Kramer's test for statistical significance of difference between mean shoot lengths of any two NaCl treatment concentrations. * against a value indicates significant difference between the effect of two concentrations.

NaCl concentrations compared	Difference between the two mean values of heights for the cultivars used in the study				
	Pokkali	Lunishree	Lunisankha	Swarna	Naveen
0-85 mM	0.76	1.44*	1.28*	1.30*	1.70*
0-170 mM	3.34*	1.52*	2.13*	2.80*	4.15*
0-256 mM	5.99*	3.67*	3.20*	4.18*	4.73*
0-342 mM	6.19*	6.78*	4.84*	4.58*	5.83*
85-170 mM	2.58*	0.08	0.85	1.50*	2.45*
85-256 mM	5.23*	2.23*	1.93*	2.87*	3.04*
256-342 mM	5.43*	5.34*	3.56*	3.28*	4.14*
170-256 mM	2.65*	2.15*	1.08*	1.37*	0.59
170-342 mM	2.84*	5.27*	2.07*	1.78*	1.68*
256-342 mM	0.20	3.11*	1.63*	0.41*	1.10*

The importance of going for short term (72 h) IC_{50} determination is that it gives an indication of the breeding lines that should be considered for salt tolerance testing by the long term exposure testing methods already available (Rekha et al., 2018; Bado et al., 2016), instead of testing all the genotypes, which may be more than 200 in numbers (Mondal and Borromeo, 2016), by long term exposure. For example, the cultivar Naveen can outright be opted out for the long term exposure testing, as its

72 h IC_{50} is 11.38 dS/m, almost equivalent to the value (10 dS/m) at which the plant dies before maturity (Munns et al., 2006). Even Swarna can be opted out of the long term exposure testing because of showing arrest in growth from 256 mM onwards, a concentration little higher than the IC_{50} value for shoot length inhibition. Screening of 231 rice genotypes for salt tolerance at the seedling stage by long term exposure method has shown that only 4 and 18 genotypes were tolerant and moderately tolerant, respectively, and the rest were sensitive to highly sensitive (Mondal and Borromeo, 2016). Initial performance of 72 h IC_{50} test for shoot inhibition could have thus allowed omission of at least 90 % of the genotype for long term exposure testing and visual scoring. Besides, according to the present method a NaCl concentration of 153 mM can be safely considered as a concentration at which a breeding line should be considered as salt-sensitive if the inhibition in its shoot growth is at least 50% in 72 h exposure period.

CONCLUSION

The study thus showed that salt tolerance in rice genotype can easily be determined by estimation of 72 h IC_{50} for shoot length of 5 days old seedlings grown on hydroponic medium. The method is sensitive enough to distinguish two genotypes with a minor difference in salt tolerance. The estimation of 72 h IC_{50} of rice seedlings on the hydroponic medium is also suitable for screening a large number (batch) of genotypes for their salt tolerance in a short time. The method is particularly suitable for screening out the salt-sensitive genotypes, leaving the salt-tolerant genotypes for further screening of their salt tolerance ability by the standard long period exposure method. The adaptation of the present short term IC_{50} based hydroponic screening protocol in the initial screening of the breeding lines for their salt tolerance would reduce the screening time greatly, and at the same time would be highly cost-effective and less labor incentive compared with the long period exposure method being followed directly.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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